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<b>(54) Title:</b> I $\kappa$ B AND ENHANCED GREEN FLUORESCENT FUSION PROTEIN  <b>(57) Abstract</b>  The present invention provides fusion protein comprising I $\kappa$ B and green fluorescent protein. Also provided is DNA encoding this protein and vector expressing such DNA and various methods of using a fusion protein comprising I $\kappa$ B and green fluorescent protein.			

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**I $\kappa$ B AND ENHANCED GREEN FLUORESCENT FUSION PROTEIN**

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**BACKGROUND OF THE INVENTION**

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Field of the Invention

The present invention relates generally to the fields of protein chemistry and biochemical assays and reagents. More specifically, the present invention relates to modified fluorescent proteins and to methods for their use.

15

Description of the Related Art

The Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* has been widely used as a reporter in the determination of gene expression and protein localization (1-4). GFP

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cDNA can be expressed in various cells or organisms with an easily detected fluorescence in the absence of any substrate or cofactor (5).

GFP is a 27-kDa, single-chain polypeptide of 238 amino acids (6). A key sequence of Ser-Tyr-Gly at amino acids 65 to 67  
5 near the N terminus functions as the GFP fluorophore (7). These three amino acids undergo spontaneous oxidation to form a cyclized chromophore responsible for the fluorescence of GFP (8). Enhanced GFP (EGFP) is a mutant of GFP with a 35-fold increase in fluorescence (9-11). This variant has mutations of Ser to Thr at amino acid 65  
10 and Phe to Leu at position 64, and is encoded by a gene with optimized human codons (10).

Because of its easily detectable green fluorescence, green fluorescent protein from the jellyfish *Aequorea victoria* has been used widely to study gene expression and protein localization. GFP  
15 fluorescence does not require a substrate or cofactor; hence, it is possible to use this reporter in numerous species and in a wide variety of cells. GFP is a very stable protein, and can accumulate; thus, GFP is often toxic to mammalian cells.

Recently, crystallographic structures of wild-type GFP and  
20 the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormo et al. (1996) *Science* 273: 1392-1395; Yang, F., Moss,

L. G., and Phillips, G. N., Jr. (1996) *Nature Biotech* 14: 1246-1251).

The barrel consists of beta sheets in a compact antiparallel structure.

In the center of the barrel, an alpha helix containing the chromophore is shielded by the barrel. The compact structure

5 makes GFP very stable under diverse and/or harsh conditions, such as protease treatment.

A great deal of research is being performed currently to improve the properties of GFP and to produce GFP reagents useful for a variety of research purposes. New versions of GFP have been  
10 developed via mutation, including a "humanized" GFP DNA, the protein product of which enjoys increased synthesis and improved folding in mammalian cells (see Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) *Gene* 173, 33-38; Haas, J., Park., E. C., and Seed, B. (1996) *Current Biology* 6, 315-324; and Yang, T. T., Cheng, L., Kain,  
15 S. R. (1996) *Nucleic Acids Research* 24, 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue- and red-fluorescent light emitting versions.

Cells are capable of responding to various stimuli by  
20 activating or repressing the expression of particular genes whose products exert a wide range of effects on biological processes. Such

stimuli include heat shock, steroid hormone, growth factors, cytokines, etc. The process of gene transcription itself is the major point for regulation, although gene expression can be regulated at the post-transcriptional level. The external signals or stimuli may  
5 affect gene expression via regulation of transcriptional factors in a process called signal transduction. External signals exert influence by, for example, inducing conformational changes in these factors, modifying them chemically, or directing formation of ligand/protein complexes. The dissection of signal transduction pathways provide  
10 important information for the drug discovery and design.

A number of important signal transduction pathways require the activation of transcription factor NF- $\kappa$ B. Compounds affecting NF- $\kappa$ B activation include tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin 1 $\beta$  (IL-1), liposaccharide (LPS), and phorbol ester (PMA).  
15 The activation of NF- $\kappa$ B leads to induction of a large array of genes whose products contribute diverse important biological processes, such as cell growth, apoptosis, inflammation, and immune responses. NF $\kappa$ B is the prototype of a family of dimeric transcription factors. It consists of p50(NF $\kappa$ B1), p52(NF $\kappa$ B2), RelA(p65), RelB, c-Rel. The  
20 Rel/NF $\kappa$ B family of proteins share an approximately 300-amino acid Rel region that binds to DNA, interacts with each other, and binds its

inhibitor, I $\kappa$ B. There are at least six species I $\kappa$ B proteins that are identified to associate with NF $\kappa$ B/Rel proteins. All I $\kappa$ B proteins have 5-7 ankyrin repeat domains, each with a length of about 30 amino acids, that interact with the NF $\kappa$ B Rel region.

5           The mechanism of NF $\kappa$ B activation has been well documented. NF $\kappa$ B is present in an inactive form in the cytoplasm where it is bound to I $\kappa$ B. Cellular activation in response to a variety of inducers leads to the rapid release of NF $\kappa$ B from I $\kappa$ B. This activation is independent on protein synthesis. In response to  
10 diverse stimuli, an intracellular cascade of protein kinase activity is triggered, and I $\kappa$ B is phosphorylated at two serines (Ser 32 and Ser 36) specifically and degraded rapidly by 26S proteasome. Uncomplexed NF $\kappa$ B rapidly translocates to the nucleus where transcriptional activation of gene expression occurs within minutes.

15           The search for agonists or antagonists that can activate or inhibit NF $\kappa$ B are major drug targets for pharmaceutical companies. Two very common methods used for studying the activation of gene expression are the in vitro gel shift assay or assays involving in vivo transcriptional induction of a reporter. Because the mechanism of  
20 NF $\kappa$ B activation is well defined, a number of assays based on the activation pathway have been designed and used to monitor NF $\kappa$ B-

induced gene expression. The assays include measuring phosphorylation of I $\kappa$ B, degradation of I $\kappa$ B, and ubiquitination of I $\kappa$ B. These assays are often used in the research labs; however, for large scale drug screening, the assays are either time consuming or  
5 tedious.

The prior art is deficient in an methods and research tools for easy, high throughput assays for measuring degradation of I $\kappa$ B. The present invention satisfies this long-standing need in the art.

10

## SUMMARY OF THE INVENTION

The fusion protein consisting of I $\kappa$ B and humanized green  
15 fluorescent protein of the present invention provides a research tool for measuring degradation of I $\kappa$ B in a straightforward manner. Cells expressing this fusion protein maintain GFP-induced fluoresce as long as the degradation of I $\kappa$ B has not been triggered. However, as soon as external factors induce phosphorylation of I $\kappa$ B triggering I $\kappa$ B  
20 degradation, the I $\kappa$ B portion of the fusion protein is degraded, and the GFP portion of the fusion ceases to fluoresce. Thus, the I $\kappa$ B-EGFP



fusion protein provides a tool for a direct, visual assay of I $\kappa$ B degradation. Such an assay is applicable in high throughput formats.

The present invention provides an I $\kappa$ BEGFP fusion protein  
5 wherein fluorescence of the fusion protein is dependent upon the I $\kappa$ B portion of the fusion protein being intact. In one embodiment, there is provided a fusion protein comprising an I $\kappa$ B fused to a GFP.

In yet another aspect of the invention, there is provided an isolated DNA molecule encoding an I $\kappa$ B/GFP fusion protein  
10 wherein fluorescence of the fusion protein is dependent upon the I $\kappa$ B portion of the fusion protein being intact. In one embodiment of this aspect of the invention, the isolated DNA molecule encoding the GFP portion of the fusion protein is humanized. Further, the present invention provides a vector capable of expressing the isolated DNA  
15 molecule encoding the I $\kappa$ B/GFP fusion protein wherein fluorescence of the fusion protein is dependent upon the I $\kappa$ B portion of the fusion protein being intact. In one embodiment of the vector, the vector contains an inducible promoter. In another aspect of the invention, there is provided a mammalian cell stably-transfected with a DNA  
20 expressing the I $\kappa$ B/GFP fusion protein of the present invention.

In yet another aspect of the invention, there are provided assays for determining agonists or antagonists that activate or inhibit NF $\kappa$ B via measuring degradation of I $\kappa$ B. In one embodiment of this aspect, there is provided a method of determining activation of NF $\kappa$ B by a compound of interest, comprising the steps of: introducing into  
5 a cell a vector expressing an isolated DNA molecule encoding an I $\kappa$ B-GFP fusion protein; contacting said cell with said compound of interest; measuring the amount of GFP-induced fluorescence, wherein a decrease in GFP-induced fluorescence by said compound  
10 indicates that said compound activates NF $\kappa$ B. Generally, the decrease in GFP-induced fluorescence is directly proportional to the degradation of I $\kappa$ B.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above  
20 may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended

drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figure 1 shows a is a schematic of a vector which expresses one embodiment of the I $\kappa$ B/GFP fusion protein of the present invention.

Figure 2 illustrates the decrease in fluorescence after treatment with TNF-alpha in Hela tet-off cells stably transfected with a vector which expresses an I $\kappa$ B/GFP fusion protein. Cells were treated with TNF-alpha and examined at 0, 10, 20 and 30 minutes. Fluorescence was observed in cells at 0 and 10 minutes, but not at 20 or 30 minutes.

Figure 3 shows the results flow cytometric analysis of Hela tet-off cells stably transfected with a vector which expresses an I $\kappa$ B/GFP fusion protein. Again, cells were treated with TNF-alpha and aliquots were tested for fluorescence at the indicated time points. Results indicate that the half life of the I $\kappa$ BEGFP fusion protein is approximately 15 minutes.

Figure 4 is a western blot of protein extracts from Hela tet-off cells stably transfected with a vector which expresses an I $\kappa$ B/GFP fusion protein, using a monoclonal antibody against GFP.

The size of the fusion protein is 65 kDa, as expected. Presence of the fusion protein declined upon treatment with TNF-alpha.

### DETAILED DESCRIPTION OF THE INVENTION

5           The present invention provides a fusion protein consisting of IκB/GFP and assays to monitor NFκB activation by determining cellular fluorescence using this fusion protein. As a result of expressing the IκB/GFP fusion protein, fluorescence in these cells corresponds directly with existence of intact IκB, which, in turn, 10 corresponds directly to the state of NFκB activation. Upon exposure of these cells to various stimuli in which degradation of IκB is induced, degradation of the GFP portion of the fusion protein is triggered as well. Degradation of the fusion protein can be measured directly by determining the change in fluorescence of transfected 15 cells.

As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *A. victoria* GFP has 20 been disclosed in Prasher D.C. et al (1992) *Gene* 111:229-33.

As used herein, the term "EGFP" refers to GFP which has been "humanized", as reported in Kain et al. (1995) *Biotechniques* 19(4):650-55. "Humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for expression of the  
5 protein in human cells.

As used herein, the term "half life" refers to the period of time in which half of the fluorescent signal from a fluorescent protein expressed in cells disappears and half remains.

As used herein, the term "humanized" refers to  
10 engineering a DNA sequence coding for a protein such that the codons are optimized for translation in higher eukaryotes.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such  
15 techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985));  
20 "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986));

"Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to  
5 bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule,  
10 and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is  
15 transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not  
20 limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA,

and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence may be located 3' to the coding sequence.

Transcriptional and translational control sequences are  
5 DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating  
10 transcription of a downstream (3' direction) coding sequence. Generally, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within  
15 the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various  
20 vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by  
5 exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With  
10 respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a  
15 population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

A "heterologous" region of the DNA construct is an  
20 identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when



the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, heterologous DNA includes coding sequence in a construct  
5 where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "promoter" refers to any nucleic  
10 acid sequence whose regulation affects expression and production of the green fluorescent protein of the present invention.

Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, which provide for  
15 the expression of a coding sequence in a host cell.

The amino acids described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained  
20 by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free

carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

5	Y	Tyr	tyrosine
	G	Gly	glycine
	F	Phe	Phenylalanine
	M	Met	methionine
	A	Ala	alanine
10	S	Ser	serine
	I	Ile	isoleucine
	L	Leu	leucine
	T	Thr	threonine
	V	Val	valine
15	P	Pro	proline
	K	Lys	lysine
	H	His	histidine
	Q	Gln	glutamine
	E	Glu	glutamic acid
20	W	Trp	tryptophan
	R	Arg	arginine
	D	Asp	aspartic acid
	N	Asn	asparagine
	C	Cys	cysteine

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All amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to

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correlate the three-letter and one-letter notations which may appear alternately herein.

The present invention discloses a fusion protein comprising I $\kappa$ B and green fluorescent protein. In one aspect, the protein is a wild type green fluorescent protein. In another aspect, the protein is a humanized green fluorescent protein. Preferably, the protein is an enhanced green fluorescent protein.

The present invention is also directed to an isolated DNA molecule encoding an I $\kappa$ B/GFP fusion protein. In addition, the present invention describes a vector capable of expressing this isolated DNA molecule. Further, the present invention is directed to a cell line transfected with this vector.

The present invention is also directed to a method of determining activation of NF $\kappa$ B by a compound of interest, comprising the steps of introducing into a cell a vector expressing an isolated DNA molecule encoding an I $\kappa$ B-GFP fusion protein; contacting said cell with said compound of interest; and measuring the amount of GFP-induced fluorescence, wherein a decrease in GFP-induced fluorescence by said compound indicates that said compound activates NF $\kappa$ B. The decrease in GFP-induced fluorescence is directly proportional to the degradation of I $\kappa$ B.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

## EXAMPLE 1

### Generation of I $\kappa$ B $\alpha$ -EGFP fusion protein

The cDNAs encoding I $\kappa$ B $\alpha$  and EGFP were amplified with Pfu DNA polymerase. I $\kappa$ B $\alpha$  was amplified with primers that incorporated a SacII recognition sequence at the 5' end and a BamH I sequence at the 3' end. The stop codon of I $\kappa$ B $\alpha$  was deleted during PCR amplification in order to make an open reading frame with EGFP. EGFP was amplified with primers that incorporated a BamH I recognition sequence at the 5' end and an EcoRI sequence at the 3' end. The amplified PCR products were ligated at the BamH I site and the resulting fusion constructs (I $\kappa$ B $\alpha$ -EGFP) were cloned into SacII and EcoRI sites of the pTRE expression vector for use in the Tetracycline (Tc)-regulated expression system (Gossen M., and Bujard H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89, 5547-5551).

## EXAMPLE 2

### Establishment of a HeLa cell line expressing the fusion protein

To make stable cell lines, DNA constructs were co-  
5 transfected into HeLa Tet-off cells with pTK-Hyg. HeLa tet-off cells are  
HeLa cells which were pre-transfected by a fusion protein of the tet-  
repressor and the herpes simplex virus VP16 (tTA). This pre-  
transfection allows expression of the gene on pTRE vector (Gossen  
and Bujard, *ibid*), which in turn initiates transcription by binding to  
10 a modified CMV promoter with tet-repressor binding elements. This  
binding can be blocked by tetracycline; hence, the expression can be  
controlled by tetracycline. The transfected cells were cultured in a  
medium containing hygromycin to select drug resistant cells. The  
drug-resistant colonies were then screened for green fluorescent  
15 clones under a fluorescence microscope. The fluorescent clones were  
selected and transferred.

## EXAMPLE 3

### 20 Fluorescence Analysis

Cells were cultured on top of cover-slips to allow  
observation under a fluorescence microscope. After transfection, the

cells were incubated at 37°C for 24 hours on the cover-slips and then fixed with 4% paraformaldehyde for 30 minutes. The cover-slips were mounted on a glass slide for fluorescence examination with a Zeiss Axioskop Model 50 fluorescent microscope. To  
5 determine protein turnover, the cells were treated with TNF for varying times before paraformaldehyde fixation. The transfected cells were collected by EDTA treatment and the cell pellets resuspended in 0.5 ml of PBS. The cell suspensions were analyzed for fluorescence intensity using a FACS Calibur flow cytometer.  
10 (Becton Dickson, Inc., San Jose, CA). EGFP was excited at 488 nm, and emission detected using a 510/20 bandpass filter.

For FACS analysis, the transfected cells were collected by EDTA treatment and the cell pellets were resuspended in 0.5 ml of PBS. The cell suspensions were then analyzed for fluorescence  
15 intensity by FACS Calibur (Becton Dickson, Inc., San Jose, CA). EGFP was excited at 488nm, and emission was detected using a 510/20 bandpass filter.

#### EXAMPLE 4

##### 20 Western Blot Analysis

For western blot analysis, transfected control cells were collected in PBS and sonicated to prepare cell lysates. Proteins were

separated by SDS-PAGE. The I $\kappa$ B $\alpha$ -EGFP fusion proteins were detected by a monoclonal antibody against GFP after the proteins were transferred onto a membrane. The detection was visualized with a chemiluminescent detection kit (CLONTECH).

5

### EXAMPLE 5

#### Characterization of I $\kappa$ B $\alpha$ EGFP

10 A stable cell line expressing the I $\kappa$ B $\alpha$ EGFP fusion protein was established by co-transfecting the pTRE-I $\kappa$ B $\alpha$ EGFP construct with a pTK-hygromycin construct, selecting the resistant colonies, and screening for fluorescence under a fluorescent microscope. Two clones were selected, and because each clone is derived from a single  
15 colony, every single cell fluoresces. This makes the observation and detection much easier.

To examine more carefully the regulation of the fluorescence, cells were treated with TNF for 0, 20, 40, 60 and 80 minutes and aliquots of cells were monitored for fluorescence  
20 intensity at various time points. Fluorescence declined rapidly after the treatment, even after 20 minutes. Thus, time for TNF treatment was shortened and the experiments were repeated. Cells were

treated for 0, 10, 20 and 30 minutes with TNF, and fluorescence was observed in cells that were treated for 0 or 10 minutes, but not cells treated for 20 and 30 minutes. These results suggest that the half life of the fluorescence of the fusion protein is about 10 to 20  
5 minutes.

Next, flow cytometry was used to analyze quantitatively the half life of the fluorescence. In this study, cells were treated with TNF for 0, 10, 20, 30, 40, and 50 minutes and collected for analysis. Fluorescence declined to a basal level after 20 minute treatment,  
10 agreeing with the observations obtained with the fluorescent microscope.

To more accurately determine fluorescence half life of the fusion protein, cells were treated with TNF for 0, 5, 10, 15 and 20 minutes. Results indicated that the half life of the fluorescence of  
15 the fusion protein is 15 minutes--very close to the half life of I $\kappa$ B protein that was determined in TNF treated Hela cells. The EGFP tag neither changes TNF-mediated regulation of I $\kappa$ B nor its half life. Therefore, the fusion protein I $\kappa$ B $\alpha$ EGFP can be used to monitor I $\kappa$ B degradation.

20 To examine if TNF-mediated IG degradation is cell growth-dependent or not, the cells of the IG clone was cultured in



regular serum medium overnight. The cells were washed and medium was replaced with serum free medium. The growth-arrested cells were then treated with TNF for 30 minutes. The fluorescence of the growth-arrested cells did not change dramatically compared to the growing cells. The fluorescence was still sensitive to the TNF treatment, suggesting that TNF-mediated IKB degradation is independent of cell growth.

#### EXAMPLE 6

10

##### Further Characterization of Ikb $\alpha$ EGFP

To confirm that the fluorescence change represents the change of protein level and not the influence of an external factor that might effect fluorescence, protein change was measured by Western blot analysis with a monoclonal antibody against recombinant GFP. The cells were treated with TNF-alpha for 0, 5, 10, 15, and 20 minutes. The molecular size of the Ikb $\alpha$ EGFP fusion protein is about 65 kDa, as was predicted. The presence of the fusion protein declined with the increase of time of TNF treatment. After 10 minutes of treatment, more than half amount of the fusion protein disappeared, confirming the results obtained measuring

fluorescence-- that the half life of the fusion protein is less than 15 minutes.

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5           Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to  
10 be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures,  
15 treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined  
20 by the scope of the claims.

**WHAT IS CLAIMED IS:**

1. A fusion protein comprising I $\kappa$ B and green  
5 fluorescent protein.
2. The green fluorescent protein of claim 1, wherein  
said protein is a wild type green fluorescent protein.
- 10 3. The green fluorescent protein of claim 1, wherein  
said protein is a humanized green fluorescent protein.
- 15 4. The green fluorescent protein of claim 1, wherein  
said protein is an enhanced green fluorescent protein.
- 20 5. An isolated DNA molecule encoding an I $\kappa$ B/GFP  
fusion protein of claim 1.

6. A vector capable of expressing the isolated DNA molecule of claim 5.

5 7. A cell line transfected with the vector of claim 6.

8. A method of determining activation of NF $\kappa$ B by a compound of interest, comprising the steps of:

10 introducing into a cell a vector expressing an isolated DNA molecule encoding an I $\kappa$ B-GFP fusion protein;

contacting said cell with said compound of interest;

measuring an amount of GFP-induced fluorescence,

wherein a decrease in GFP-induced fluorescence by said compound

15 indicates that said compound activates NF $\kappa$ B.

9. The method of claim 8, wherein a decrease in GFP-induced fluorescence is directly proportional to the degradation of I $\kappa$ B.

20

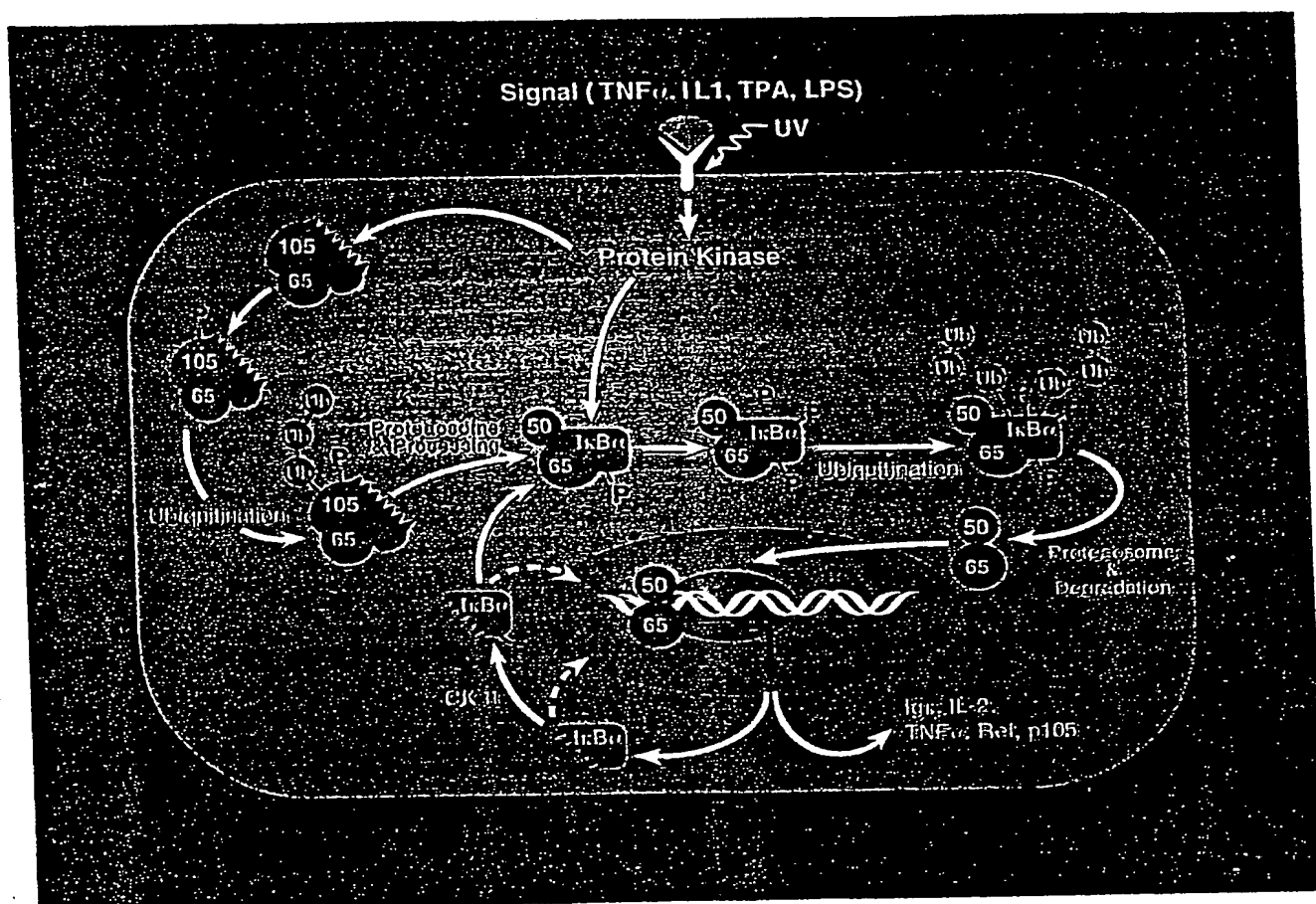


Figure 1



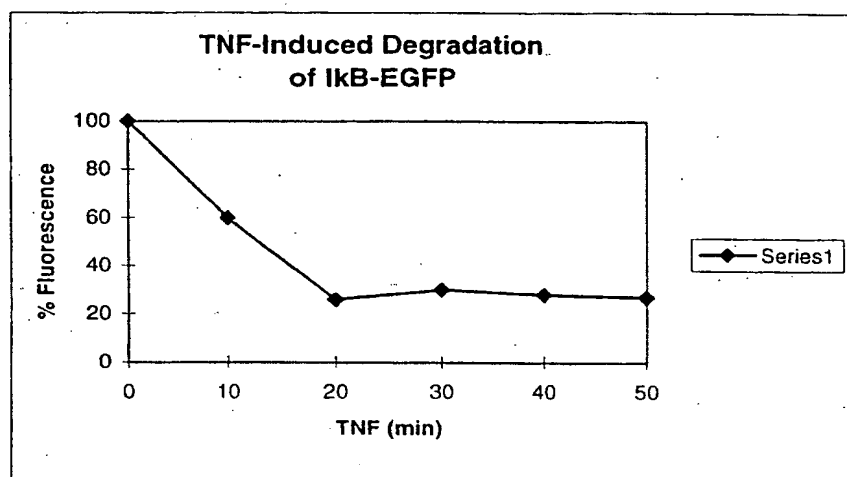
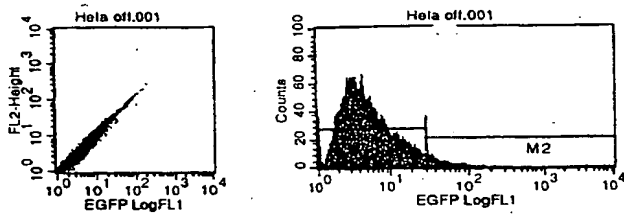


Figure 2

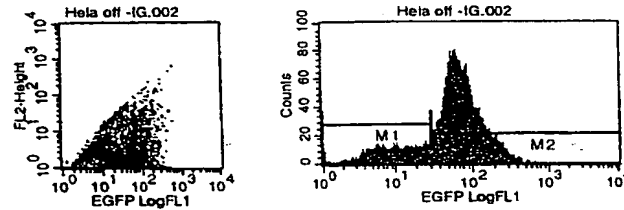


File: HeLa off.001

Acquisition Date: 19-Dec-97

Total Events: 10000

Marker	Left	Right	Events	% Total	Mean
All	1	9910	10000	100.00	7.17
M1	1	30	9704	97.04	5.96
M2	30	9910	299	2.99	46.78



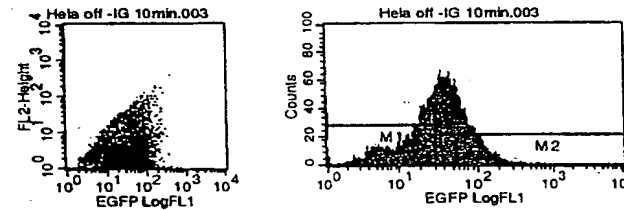
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Acquisition Date: 19-Dec-97

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All	1	9910	10000	100.00	70.72
M1	1	30	1924	19.24	12.34
M2	30	9910	8090	80.90	84.54

M2 All  
100% 100



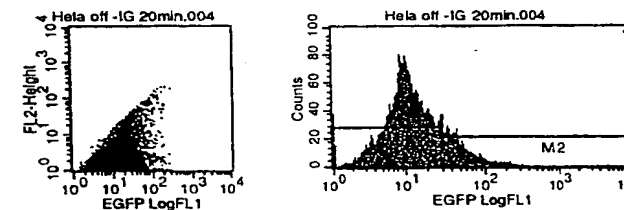
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Acquisition Date: 19-Dec-97

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All	1	9910	10000	100.00	42.40
M1	1	30	4168	41.66	16.98
M2	30	9910	5882	58.82	60.30

73% 60%



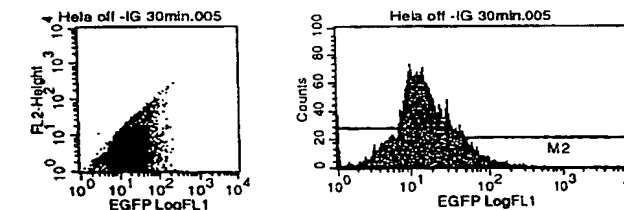
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Acquisition Date: 19-Dec-97

Total Events: 10000

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All	1	9910	10000	100.00	18.00
M1	1	30	8516	85.16	11.63
M2	30	9910	1502	15.02	54.21

19% 26%



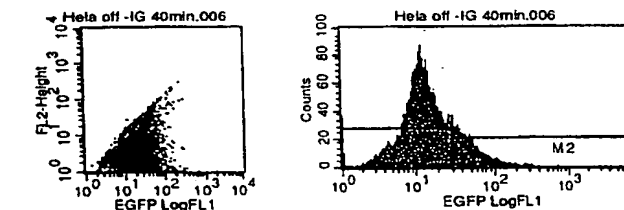
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Acquisition Date: 19-Dec-97

Total Events: 10000

Marker	Left	Right	Events	% Total	Mean
All	1	9910	10000	100.00	20.88
M1	1	30	8191	81.91	13.20
M2	30	9910	1835	18.35	55.27

22% 30%



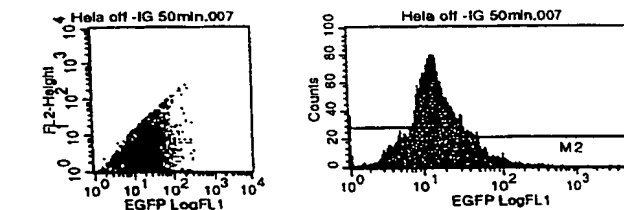
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Acquisition Date: 19-Dec-97

Total Events: 10000

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M1	1	30	8325	83.25	12.89
M2	30	9910	1702	17.02	54.83

21% 28%



File: HeLa off -IG 50min.007

Acquisition Date: 19-Dec-97

Total Events: 10000

Marker	Left	Right	Events	% Total	Mean
All	1	9910	10000	100.00	19.23
M1	1	30	8497	84.97	13.16
M2	30	9910	1529	15.29	53.14

19% 27%

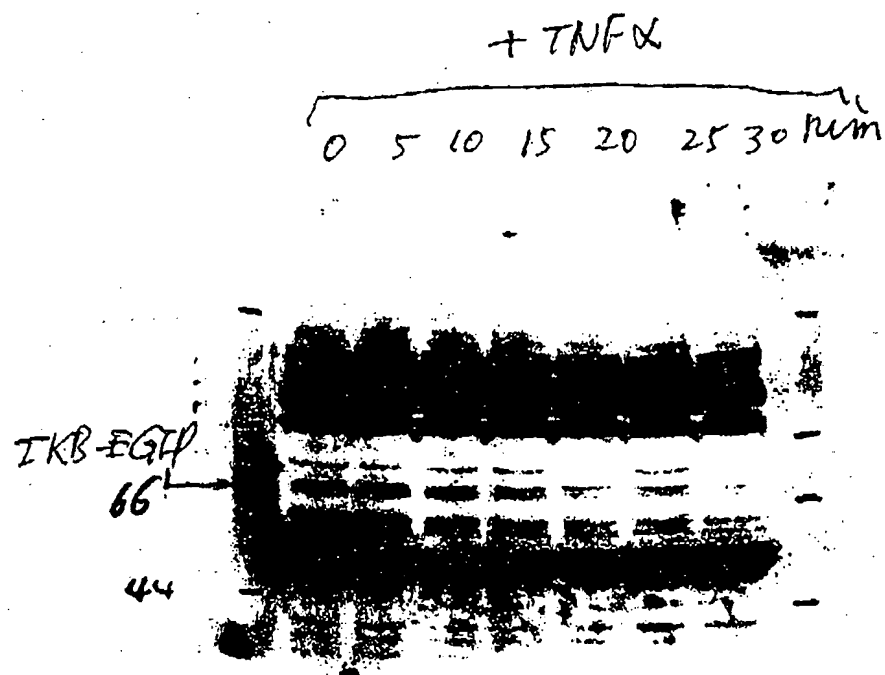


Figure 4

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/08366**A. CLASSIFICATION OF SUBJECT MATTER**IPC(6) : G01N 33/53; C07K 1/00  
US CL : 435/7.1, 7.21, 69.7; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.21, 69.7; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
APS, MEDLINE, BIOSIS, DIALOG, STN**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,597,898 A (GHOSH) 28 January 1997, Examples 1, 2, 5,8,9; Column 14, Lines 44-49.	1-4
Y	US 5,846,714 A (HASKILL et al) 08 December 1998, Example 5.	1-4
Y	YANG et al. Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic acid research. 1996, Vol. 24, No. 22, pages 4592-4593, see entire document.	1-4
Y	CORMACK et al. FACS-Optimized mutants of the green fluorescent protein (GFP). Gene. 1996. Vol 173, pages 33-38, especially pages 34-38.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

13 AUGUST 1999

Date of mailing of the international search report

09 SEP 1999

Name and mailing address of the ISA/US  
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Authorized officer

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Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/08366

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/08366

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I claim(s) 1-4, drawn to a fusion protein comprising I $\kappa$ B and green fluorescent protein.

Group II, claim(s) 5-9, drawn to a DNA molecule encoding an I $\kappa$ B/GFP fusion protein and method of use.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn to a fusion protein of I $\kappa$ B/GFP which is structurally and functionally different from DNA of Group II. Therefore, they don't share a common core structure